

Effect of a Single Treatment with Keishininjinto on Plasma Levels of Gut-regulatory Peptides in Healthy Subjects

Yuhki Sato,* Fumihiko Katagiri, Shin Inoue, Hiroki Itoh, and Masaharu Takeyama

Department of Clinical Pharmacy, Oita University Hospital, 1-1 Idaigaoka, Hasama-machi, Oita 879-5593, Japan.

(Received October 17, 2006; Accepted December 29, 2006; Published online January 19, 2007)

A traditional Japanese Kampo medicine Keishininjinto has been empirically used for the treatment of headache, chronic gastroenteritis, gastric atony, and watery diarrhea often accompanying abdominal pain, cold, fever, and headache. One of the mechanisms of the empirical effects is assumed to be due to local changes in gut-regulated peptide levels. We studied the effects of Keishininjinto on calcitonin gene-related peptide (CGRP)-, substance P-, vasoactive intestinal polypeptides (VIP)-, motilin-, and somatostatin-like immunoreactive substances (IS) in plasma taken from healthy subjects. Keishininjinto (6.0g) or placebo was orally administered to five healthy males. Blood samples were taken before, and at 20, 40, 60, 90, 120, 180, and 240 min after administration, followed by the extracting procedure, and submitted to a highly sensitive enzyme immunoassay system for CGRP-, substance P-, VIP-, motilin-, and somatostatin-IS. A single oral administration of Keishininjinto caused significant increases in plasma CGRP-, and substance P-IS levels compared with placebo. On the other hand, a single oral administration of Keishininjinto transiently caused decreases in plasma VIP-IS levels compared with placebo. However, Keishininjinto had no effects on plasma motilin-, and somatostatin-IS levels. In this study, we hypothesized that Keishininjinto might stimulate capsaicin-sensitive afferent nerves and improve gastrointestinal mucosal blood flow, and that might affect intestinal secretion and motility in neuronal reflexes.

Key words — Keishininjinto, calcitonin gene-related peptide, substance P, vasoactive intestinal polypeptides,

gastrointestinal mucosal blood flow, diarrhea

INTRODUCTION

Kampo (Japanese herbal) medicines (Ninjinto, Daikenchuto, and Rikkunshito, *etc.*) are clinically used to treat chronic hypofunction of the gastrointestinal system. In recent years, some Kampo medicines used to treat those experimental gastrointestinal diseases have been elucidated pharmacologically from the viewpoint of gut-regulated hormone levels. One of them, Daikenchuto is known to increase gastrointestinal motility and blood flow, and improve ileal function.¹⁾ Furthermore, these effects are reported to cause significant increases in the levels of gut-regulated peptides such as motilin, vasoactive intestinal peptides (VIP), calcitonin gene-related peptide (CGRP), and substance P in human plasma.²⁻⁴⁾ Ninjinto is also reported to enhance gastrointestinal motility, similar to the gastrointestinal prokinetic drugs like cisapride and metoclopramide, and to be an effective herbal medicine for postoperative ileus.⁵⁾ These effects were reported to cause significant increases in the levels of motilin, somatostatin, CGRP, and substance P in human plasma.^{6,7)}

Keishininjinto, a traditional Japanese medicines, is prepared from five crude drugs; *Cinnamomi cortex*, *Ginseng radix*, *Glycyrrhizae radix*, *Atractylodis rhizome*, and *Zingiberis siccatum rhizome*. In Kampo medical care, this formula is traditionally used to treat chronic hypofunction of the gastrointestinal tract, including gastroenteritis, gastric atony, gastrectasis, nausea, and vomiting. In addition to these effects, it is thought that Keishininjinto empirically has the pharmacologic effects for watery and infectious diarrhea often accompanying abdominal pain, cold, fever, and headache. We hypothesized that the pharmacologic effect of Keishininjinto might be due to changes in gut-regulated peptides levels. Hence, we examined the temporal effects of Keishininjinto on CGRP-, substance P-, VIP-, motilin-, and somatostatin-like immunoreactive substances (IS) levels in plasma as measured by a high sensitive enzyme immunoassay (EIA) in healthy subjects.

*To whom correspondence should be addressed: Department of Clinical Pharmacy, Oita University Hospital, 1-1 Idaigaoka, Hasama-machi, Oita 879-5593, Japan. Tel.: +81-97-586-6112; Fax: +81-97-586-6119; E-mail: syuhki@med.oita-u.ac.jp

MATERIALS AND METHODS

Materials — Keishininjinto (EK-82), prepared as a 6.0 g dried powdered extract in the proportion: *Cinnamomi cortex* (4.0 g), *Ginseng radix* (3.0 g), *Glycyrrhizae radix* (3.0 g), *Atractylodis rhizoma* (3.0 g), *Zingiberis siccatum rhizoma* (2.0 g), was kindly supplied by Kanebo Co. Ltd. (Tokyo, Japan). The placebo was excipient alone (crystalline cellulose and lactose) for the above formulations. Synthetic human motilin, somatostatin, VIP, CGRP and its fragment (8–37), substance P, were purchased from the Peptide Institute (Osaka, Japan). The VIP fragment peptide was supplied by Professor H. Yajima (Kyoto University, Kyoto, Japan). Antisera to VIP (A604/R1B) and CGRP (CA1132) were purchased from Biogenesis (Poole, U.K.), somatostatin (T-4101) from Peninsula Laboratories (San Carlos, CA, U.S.A.), motilin (Y121) from Yanaihara Institute (Shizuoka, Japan) and substance P (RA-08-095) from Cambridge Research Biochemicals (Cambridge, U.K.). Goat affinity-purified antibody to rabbit IgG (whole molecule) (55641) was purchased from ICN Pharmaceuticals (Aurora, OH, U.S.A.). All other reagents were of analytical reagent grade from commercial sources.

Subjects — Five healthy male subjects (non-smokers), aged 24–29 years (median 26 years), weighing 55–68 kg (median weight 62 kg), participated in this study. Each subjects received information about the study's scientific purpose, which was approved by the Ethics Committee of Oita Medical University, and gave informed consent. No subject had received any medication for 1 month preceding the test and no stimulator of gastrointestinal motility was administered to any subject during the study.

Study Schedule — Keishininjinto (6.0 g) or placebo was orally administered with 100 ml water. Each subjects was administered these drugs with an interval of four weeks. The dose of Keishininjinto used in this study was the maximum daily dose used in clinical therapy. Venous blood samples (10 ml) from a forearm vein just before the drug was administered, at 20, 40, 60, 90, 120, 180, and 240 min after administration of the test substances. The study was carried out from 14:00 to 18:00.

Preparation of Plasma Extracts — The blood samples were collected in a chilled tube containing aprotinin (500-kallikrein inhibitor units/ml) and ethylenediaminetetraacetic acid (EDTA) (1.2 mg/ml). After centrifugation, the plasma samples were diluted with 4% acetic acid (pH 4.0)

and loaded onto Sep-Pak C₁₈ cartridges (Millipore Corp., Milford, MA, U.S.A.), and washed with 4% acetic acid. The peptides in the plasma were eluted with 70% acetonitrile in 0.5% acetic acid (pH 4.0), lyophilized, and reconstituted to 100 μ l with the assay buffer and subjected to EIA. For the EIA system, plasma samples were concentrated five-fold with Sep-Pak C₁₈ cartridges. The recovery of plasma CGRP-, substance P-, VIP-, motilin-, and somatostatin-IS was >90% with this extraction procedure.

EIAs of, CGRP, Substance P, VIP, Motilin, and Somatostatin — EIAs for CGRP-,⁸⁾ substance P-,⁹⁾ VIP-,¹⁰⁾ motilin-,¹¹⁾ and somatostatin-IS¹²⁾ were performed as previously described, by a delayed-addition method.¹³⁾ Separation of bound and free antigen was performed on an anti-rabbit IgG-coated immunoplates. The fluorescent product 4-methylumbelliferon was measured with an MTP-100F microplate reader (Corona Electric, Ibaraki, Japan). Human somatostatin, porcine motilin, fragment VIP (11–28), human CGRP (8–37) and substance P were conjugated with β -D-galactosidase (Boehringer Mannheim, Mannheim, Germany) with *N*-(ϵ -maleimidocaproyloxy)-succinimide according to the method Kitagawa *et al.*¹⁴⁾ The EIAs for somatostatin, motilin, VIP, CGRP, and substance P was specific and highly sensitive to detection limits of 0.10, 0.80, 1.00, 0.08 and 0.40 fmol/well, respectively.

Statistical Analysis — All values are expressed as the mean \pm S.D. The statistical significance evaluated by analysis of variance for repeated measure and Dunnett's test. Value of $p < 0.01$ or $p < 0.05$ was considered to represent a statistically significant difference.

RESULTS AND DISCUSSIONS

The abnormality of gastrointestinal function is caused by an obstruction of the automatic nervous system and abnormal hormone levels. In this study, gut-regulated peptide (motilin, somatostatin, VIP, CGRP, and substance P) levels, which regulated gastrointestinal function, were examined to study their relation with Keishininjinto.

The plasma CGRP-IS level-time profile after administration of Keishininjinto to healthy subjects is shown in Fig. 1 (A). Keishininjinto significantly increased CGRP-IS at 90 min (52.3 ± 22.3 pg/ml)

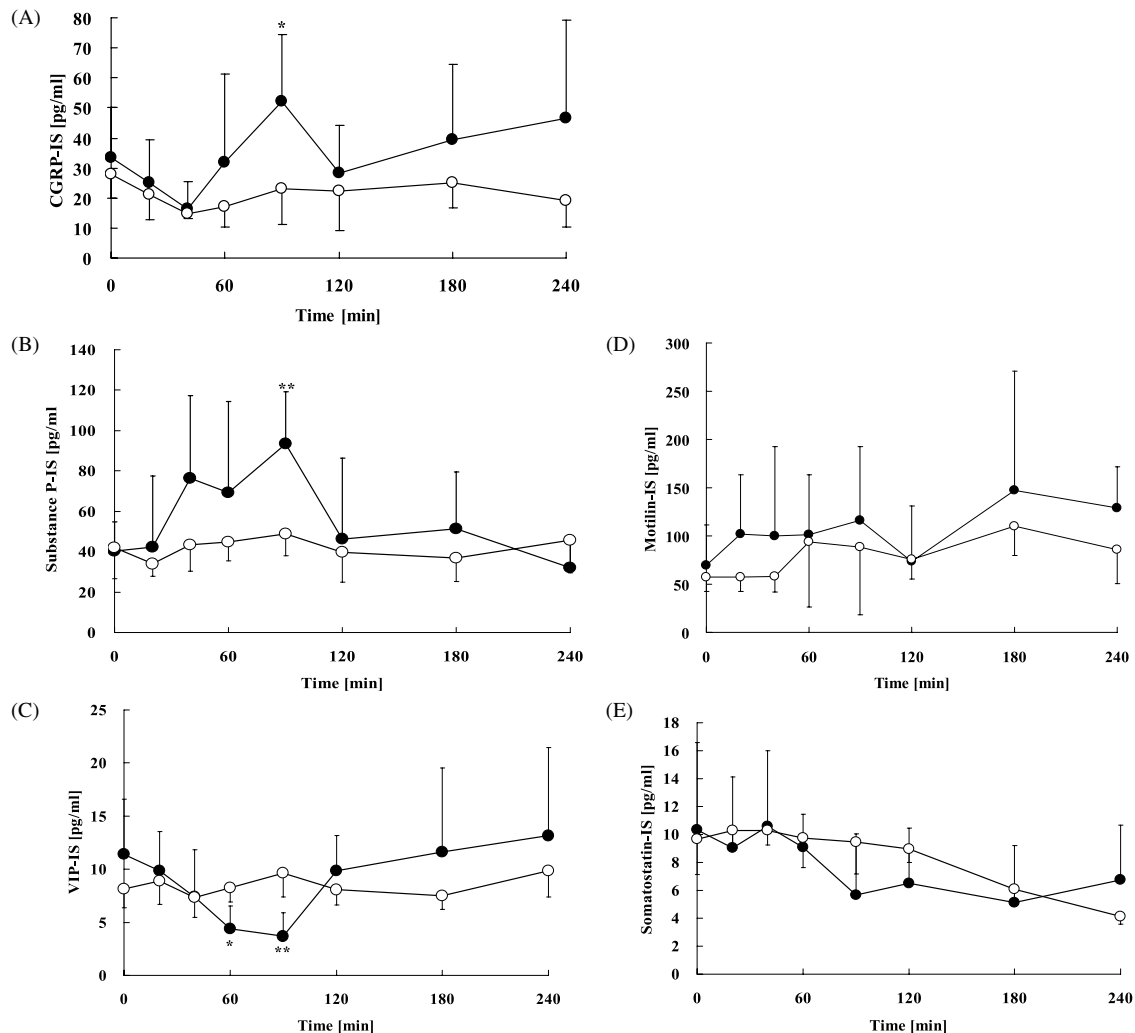


Fig. 1. Effect of Plasma CGRP (A)-, Substance P (B)-, VIP (C)-, Motilin (D)-, and Somatostatin (E)-Immunoreactive Substance (IS) Levels after an Oral Administration of Bushirichuto 6.0 g (●) or Placebo (○)
Each point represents the mean \pm S.D. of five subjects. ** $p < 0.01$ and * $p < 0.05$ compared with placebo.

compared with the response of the placebo group (23.0 ± 11.8 pg/ml). Figure 1 (B) shows plasma substance P-IS levels after administration of Keishininjinto to healthy subjects. Keishininjinto significantly increased substance P-IS at 90 min (93.5 ± 25.7 pg/ml) compared with placebo (48.8 ± 10.7 pg/ml). On the other hands, Keishininjinto transiently decreased VIP-IS at 60 and 90 min (4.4 ± 2.2 pg/ml at 60 min, and 3.7 ± 2.2 pg/ml at 90 min) compared with placebo (8.3 ± 1.4 pg/ml at 60 min, and 9.7 ± 2.3 pg/ml at 90 min) [Fig. 1 (C)]. Keishininjinto did not, however, alter levels of motilin and somatostatin [Fig. 1 (D) and (E)].

CGRP has several potent biological activities, including vasodilation, and in the gastric mucosa its vasodilatory effects following stimulated release from the extrinsic sensory innervation is consid-

ered to serve as an important protective mechanism for maintaining gastric mucosal integrity.^{15, 16)} CGRP is a potent gastrointestinal vasodilator and causes increases in the gastrointestinal mucosal blood flow.¹⁷⁾ Substance P coexists with CGRP in the sensory afferent neurons in the gastrointestinal mucosa.¹⁸⁾ Substance P also has been shown to increase in intestinal mucosal blood flow in humans.¹⁹⁾ Both CGRP and substance P are the major components of the afferent peptidergic innervation of the gastrointestinal tract, and are widely distributed around blood vessels.²⁰⁾ Various injuries factors such as neurotoxic capsaicin, which is the pungent ingredient in red peppers and a vanilloid receptor agonist, stimulates afferent sensory neurons called "capsaicin-sensitive afferent neurons," which release CGRP and substance P from

their nerve endings.¹⁹⁾ These peptides enhance mucosal resistance to injury via formation of vasodilation and hyperemia-independent mechanisms. The pathophysiological potential of this emergency system plays an important role in gastrointestinal cytoprotection.²¹⁾ In this study, Keishininjinto raised plasma CGRP- and substance P-IS levels at the same time. Keishininjinto has *Zingiberis siccaturum rhizoma* as one of its ingredients, and this herb contains 6-gingerol and 6-shogaol as bioactive components, both of which have vanilloid structures. Daikenchuto, *Zingiberis siccaturum rhizoma* and 6-shogaol produces an increase in intestinal blood flow, and the CGRP receptor antagonist completely abolished the reaction as shown by effect elicited *in vivo*.²²⁾ Naito *et al.* also reported that plasma CGRP and substance P levels significantly increased after administration of *Zingiberis rhizoma* extract to healthy subjects.²³⁾ Keishininjinto might directly or indirectly stimulate capsaicin-sensitive afferent nerves and increase the gastrointestinal mucosal blood flow by significantly increasing CGRP and substance P-IS levels in plasma.

VIP is widely distributed in the central and peripheral nervous system. This peptide has a vasodilating effect and is an important neurotransmitter for the enteric nervous system. VIP is known as a major regulator of mammalian intestinal motility and induces relaxation of precontracted ileal longitudinal muscle, and mediates the peristaltic reflex.^{24, 25)} In addition, VIP plays an important role for the nervous control of the intestinal fluid secretion.²⁶⁾ In general, diarrhea occurs when bowel contents are passed without being sufficiently digested or absorbed due to accelerated enteric juice secretion or peristalsis after stimulation of the enteric tract.^{27, 28)} The intestinal fluid secretion by VIP is altered during this condition. In our results, Keishininjinto altered plasma VIP-IS levels. Although further investigations are necessary, Keishininjinto might act in the gastrointestinal system, and part of its antidiarrheal action might be closely related to changes in VIP-IS levels in plasma.

In previous reports, Naito *et al.* assumed that Ninjinto increased plasma somatostatin and motilin levels to improve gastrointestinal motor dysfunction and intracellular communication between somatostatin and motilin levels.⁶⁾ Keishininjinto had temporally altered plasma VIP-IS level, but had no effect on plasma motilin and somatostatin levels. Tashiro reported that Ninjinto enhanced the gas-

trointestinal motility in mouse, but Keishininjinto had no effect.²⁹⁾ These results might be related to change in plasma somatostatin and motilin levels. Somatostatin, a polypeptide widely distributed in the gastrointestinal tract, participates in the control of gut motility by exerting both inhibitory and stimulating influences. In the interdigestive state somatostatin induces phase-3 activity and in the digestive state it inhibits gastric emptying and slows gastrointestinal transit.^{30, 31)} Motilin is one of the most important factors controlling the regular occurrence of phase-3 contractions of the migrating motor complex (MMC). Motilin participates in regulating gastrointestinal motility with somatostatin, and stimulates gastric emptying and postprandial gastric contraction.³²⁾

In conclusion, we have revealed that a single administration of Keishininjinto caused significant increases in plasma CGRP-, and substance P-IS levels, and altered plasma VIP-IS levels compared with placebo. Although it is necessary to examine the effects of Keishininjinto in patients with gastrointestinal diseases, we thought that Keishininjinto might indirectly stimulate capsaicin-sensitive afferent nerves and increase the gastrointestinal mucosal blood flow by significantly increasing CGRP and substance P-IS levels in plasma, and affect intestinal secretion and motility in neuronal reflexes by VIP.

REFERENCES

- 1) Seki, M. (1987) Experiences in the treatment with Chinese herb medicines for postoperative intestinal adhesion and some consideration on determining its surgical indication (Abstract in English). *Wakan-Yaku*, **4**, 94–99.
- 2) Nagano, T., Itoh, H. and Takeyama, M. (1999) Effect of Dai-kenchu-to on levels of 3 brain-gut peptides (motilin, gastrin and somatostatin) in human plasma. *Biol. Pharm. Bull.*, **22**, 1131–1133.
- 3) Nagano, T., Itoh, H. and Takeyama, M. (2000) Effects of Dai-kenchu-to on levels of 5-hydroxytryptamine (serotonin) and vasoactive intestinal peptides in human plasma. *Biol. Pharm. Bull.*, **23**, 352–353.
- 4) Sato, Y., Katagiri, F., Inoue, S., Itoh, H. and Takeyama, M. (2004) Dai-kenchu-to raises levels of calcitonin gene-related peptide and substance P in human plasma. *Biol. Pharm. Bull.*, **27**, 1875–1877.
- 5) Sekiya, M. and Morimoto, Y. (2003) Effects of sev-

- eral Kampo medicines on postoperative ileus model. *Jpn. J. Oriental Med.*, **54**, 785–790.
- 6) Naito, T., Itoh, H., Nagano, T. and Takeyama, M. (2001) Effects of Ninjin-to on levels of brain-gut peptides (motilin, vasoactive intestinal peptide, gastrin, and somatostatin) in human plasma. *Biol. Pharm. Bull.*, **24**, 194–196.
 - 7) Sato, Y., Katagiri, F., Inoue, S., Itoh, H. and Takeyama, M. (2004) Effects of Ninjin-to on levels of calcitonin gene-related peptide and substance P in human plasma. *Biol. Pharm. Bull.*, **27**, 2032–2034.
 - 8) Nagano, T., Ikawa, K. and Takeyama, M. (1998) Enzyme immunoassay of calcitonin gene-related peptide-like immunoreactive substance in human plasma and saliva. *Jpn. J. Hosp. Pharm.*, **24**, 363–369.
 - 9) Takeyama, M., Mori, K., Takayama, F., Kondo, K., Kitagawa, K. and Fujii, N. (1990) Enzyme immunoassay of a substance P-like immunoreactive substance in human plasma and saliva. *Chem. Pharm. Bull.*, **38**, 3494–3496.
 - 10) Takeyama, M., Wakayama, K., Takayama, F., Kondo, K., Kitagawa, K., Fujii, N. and Yajima, H. (1990) Micro-enzyme immunoassay of vasoactive intestinal polypeptide (VIP)-like immunoreactive substance in bovine milk. *Chem. Pharm. Bull.*, **38**, 960–962.
 - 11) Naito, T., Itoh, H. and Takeyama, M. (2002) Hange-shashin-to raises levels of somatostatin, motilin and gastrin in the plasma of healthy subjects. *Biol. Pharm. Bull.*, **25**, 327–331.
 - 12) Katagiri, F., Inoue, S., Sato, Y., Itoh, H. and Takeyama, M. (2005) Ecabet sodium raises plasma levels of calcitonin gene-related peptide and substance P in healthy humans. *J. Pharm. Pharmacol.*, **56**, 557–561.
 - 13) Takeyama, M., Kondo, K., Hayashi, Y. and Yajima, H. (1989) Enzyme immunoassay of gastrin releasing peptide (GRP)-like immunoreactivity in milk. *Int. J. Pept. Protein Res.*, **34**, 70–74.
 - 14) Kitagawa, T., Shimozone, T., Aikawa, T., Yoshida, T. and Nishimura, H. (1981) Preparation and characterization of hetero-bifunctional cross-linking reagents for protein modifications. *Chem. Pharm. Bull.*, **29**, 1130–1135.
 - 15) Tache, Y., Pappas, T., Lauffenburger, M., Goto, Y., Walsh, J. H. and Debas, H. (1984) Calcitonin gene-related peptide: potent peripheral inhibitor of gastric acid secretion in rats and dogs. *Gastroenterology*, **87**, 344–349.
 - 16) Katsoulis, S. and Conlon, J. M. (1989) Calcitonin gene-related peptides relax guinea pig and rat gastric smooth muscle. *Eur. J. Pharmacol.*, **162**, 129–134.
 - 17) Nakamura, T., Naruse, S., Ozaki, T. and Kumada, K. (1996) Calcitonin gene-related peptide is a potent intestinal, but not gastric, vasodilator in conscious dogs. *Regul. Pept.*, **65**, 211–217.
 - 18) Pawlik, W. W., Obuchowicz, R., Biernat, J., Sendur, R. and Jaworek, J. (2000) Role of calcitonin gene related peptide in the modulation of intestinal circulatory, metabolic, and myoelectric activity during ischemia/reperfusion. *J. Physiol. Pharmacol.*, **51**, 933–942.
 - 19) Schmidt, P., Lördal, M., Gazelius, B. and Hellström, P. M. (2003) Tachykinins potently stimulate human small bowel blood flow: a laser Doppler flowmetry study in humans. *Gut*, **52**, 53–56.
 - 20) Sharkey, K. A., Williams, R. G. and Dockray, G. J. (1984) Sensory substance P innervation of the stomach and pancreas. Demonstration of capsaicin-sensitive sensory neurons in the rat by combined immunohistochemistry and retrograde tracing. *Gastroenterology*, **87**, 914–921.
 - 21) Holzer, P. (1991) Capsaicin: cellular targets, mechanisms of action, and selectivity for thin sensory neurons. *Pharmacol. Rev.*, **43**, 143–195.
 - 22) Murata, P., Kase, Y., Ishige, A., Sasaki, H., Kurosawa, S. and Nakamura, T. (2002) The herbal medicine Dai-kenchu-to and one of its active components [6]-shogaol increase intestinal blood flow in rats. *Life Sci.*, **70**, 2061–2070.
 - 23) Naito, T., Itoh, H. and Takeyama, M. (2003) Comparison of the effects of Hange-shashin-to and Rikkunshi-to on human plasma calcitonin gene-related peptide and substance P levels. *Biol. Pharm. Bull.*, **26**, 1104–1107.
 - 24) Ekblad, E. and Sundler, F. (1997) Distinct receptors mediate pituitary adenylate cyclase-activating peptide- and vasoactive intestinal peptide-induced relaxation of rat ileal longitudinal muscle. *Eur. J. Pharmacol.*, **334**, 61–67.
 - 25) Grider, J. R. (2003) Neurotransmitters mediating the intestinal peristaltic reflex in the mouse. *J. Pharmacol. Exp. Ther.*, **307**, 460–467.
 - 26) Hubel, K. A. (1985) Intestinal nerves and ion transport: stimuli, reflexes, and responses. *Am. J. Physiol.*, **248**, 261–271.
 - 27) Thompson, W. G. (1984) The irritable bowel. *Gut*, **25**, 305–320.
 - 28) Narducci, F., Snape, W. J., Battle, W. M., London, R. L. and Cohen, S. (1985) Increased colonic motility during exposure to a stressful situation. *Dig. Dis. Sci.*, **30**, 40–44.
 - 29) Tashiro, S. (1994) Pharmacology of Kampo medicines. A separate volume Kampo therapy, In *IGAKU NO AYUMI* (Fujita, K., Ed.), Ishiyaku Pub-

- lishers, Inc., Tokyo, Japan, pp. 17–21.
- 30) Hostein, J., Janssens, J., Vantrappen, G., Peeters, T. L., Vandeweerdt, M. and Leman, G. (1984) Somatostatin induces ectopic activity fronts of the migrating motor complex via a local intestinal mechanism. *Gastroenterology*, **87**, 1004–1008.
- 31) Seal, A., Yamada, T., Debas, H., Hollinshead, J., Osadchey, B., Aponte, G. and Walsh, J. (1982) Somatostatin-14 and -28: clearance and potency on gastric function in dogs. *Am. J. Physiol.*, **243**, G97–G102.
- 32) Itoh, Z., Honda, R., Hiwatashi, K., Takeuchi, S., Aizawa, L., Takayanagi, R. and Couch, E. F. (1976) Motilin-induced mechanical activity in the canine alimentary tract. *Scand. J. Gastroenterol. Suppl.*, **39**, 93–110.